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THE BOTANICAL REVIEW

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TECHNIQUES FOR THE ISOLATION OF SINGLE MICROORGANISMS. II[1].

E. M. HILDEBRAND
College Station, Texas

INTRODUCTION

When the first review (Hildebrand, 1938) was prepared, emphasis was placed on isolation techniques, with particular reference to single microorganisms. Since then this phase of micrurgical science has been broadened, and, while emphasis in this article is still on the techniques of isolation, some attention will be paid to its logical sequents—inoculation and germ-free culture of microorganisms and of macroscopic organisms.

It is precisely true that when studying the mechanism of infection, inoculation is the counterpart of isolation. The inoculum must be controlled and be definite in quality and quantity, and it is conditioned by isolation technique. In this related field of interest it was demonstrated for the first time (Hildebrand, 1937) that a single isolated cell of the fire blight organism, *Erwinia amylovora*, is adequate and capable of inducing infection of apple and pear blossoms when introduced by means of a micropipette. By a modification of the same precision technique (Hildebrand, 1941, 1942), crown gall infections, caused by *Agrobacterium tumefaciens*, were readily induced by inserting or planting a single bacterial cell into small wounds on tomato. By still another technique of microinjection into living tomato plant cells it was demonstrated, on the one hand (Hildebrand, 1943), that crown-gall bacteria are incapable of inducing infection when inside the cells, whereas when aucuba mosaic virus extract was injected into single living cells of tomato plants infections resulted in as many as 50 percent of the trials. Thus inoculations in the cases cited were the sequents of isolation and in the science of cytopathology are dependent on the perfection of isolation techniques.

[1] Supplement to article in The Botanical Review 4: 627–664. 1938.

In the science of micrurgy the germ-free culture of multicellular, macroscopic organisms is a recent outstanding development that has been initiated and largely developed at Lobund,[2] the Laboratories of Bacteriology at the University of Notre Dame. The papers presented at the first symposium on Micrurgical and Germ-free Methods held there in 1939 were published subsequently (Reyniers, 1943). Just prior to the publication of this report, Baker and Ferguson (1942) announced the success of an operative procedure on pregnant platyfish (*Platypoecilus maculatus*) that regularly yielded young free from contamination.

Reyniers and associates (Reyniers, 1943; Reyniers and Trexler, 1942, 1943) have employed the term "germ-free" for the growth of vertebrate animals free from all forms of microscopic and macroscopic contaminants, whether internal or external. Subsequently, Baker and Ferguson (1942, 1944) chose the term "axenic" from the Greek, which means "free from strangers", for their cultivation of platyfish for four months free from contamination. It is up to the reader to choose which term—"germ-free" or "axenic"—best describes "life without germs" for microscopic or macroscopic plants or animals. The writer prefers "germ-free" because of its prior use and simplicity. Germ-free animals provide the best means for developing an understanding of disease resistance and immunity in animals.

MICRURGY—A NEW SCIENCE

"Micrurgy" is the term for work on a microscopic scale (Hildebrand, 1943). Thus single-cell isolation technique is a phase of micrurgical science involving micromanipulative technique under a microscope. Two popular accounts have been published about this new science (Chambers, 1935; Battista, 1947). The former article stresses the fact that there is a practicable way of studying what goes on in living cells by microvivisection, and that this is accomplished with the aid of minute needles and pipettes of glass controlled by a micromanipulator. These fine microinstruments are so tiny they are invisible except under the microscope. In most arrangements the microinstruments work against

[2] The first of the Lobund Reports (No. 1: 115 pp.) was published in 1946; its title is "Germ-free life studies". The second, entitled "The need for a nomenclature describing germ-free life and the techniques for maintaining it", was published in 1949.

the underside of the microscope slide which forms the top part of the moist chamber.

Battista (1947) characterizes micrurgy as a comparatively new science involving the use of midget tools under the microscope for delving into the fascinating worlds of the infinitely small. He predicts on the basis of present progress that one day in this atomic age it may become a major science comparable to astronomy.

The Symposium on Micrurgical and Germ-free Methods (Reyniers, 1943) has charted some of the earlier developments in micrurgy in application to experimental biology and medicine. Much of the work delineated by Battista (1947) is in application to the physical sciences—a field of interest that has not been reviewed in any literature that has come to my attention.

Reyniers (1942) and Reyniers and Trexler (1942) have further enlarged upon another phase, that is, the control of cross contamination by mechanical barriers which revamp our basic concepts on the application of microbiology to medicine.

PURE CULTURE BY DILUTION METHODS

The pure-culture concept that a culture to be pure must consist "of individuals of which we know with certainty that all are descended from one single cell, and from one only" has been occasionally challenged.

The inadequacy of the poured plate technique advocated by McNew (1938*a*, 1938*b*) for procuring pure single-cell cultures was discussed by Riker and Baldwin (1939). In brief, it was pointed out that the ordinary poured plate technique, while generally useful, appears inadequate for critical bacteriological work, particularly where strain variations are involved. The common poured-plate technique is considered insufficient because: (*a*) unless special precautions are taken, many of the colonies which develop come from bacterial clumps; (*b*) a large percentage of the colonies may run together in commonly employed dilution; while many such mixtures are readily seen, doubtless a number of others are not visible; (*c*) practical experience has shown that important plant bacteria are not always separated by dilution plates, even with elaborate precautions.

Chapman (1945) gives his concept of a "strain" in bacteriology

with the suggestion that strains "should only be considered as such when it is known that they are genetically unrelated". As was pointed out by Hildebrand (1945), our present knowledge and methods are inadequate for achieving such precision. The word "strain" usually connotes a physiological difference which is possibly genetic in origin but not feasible of ready demonstration as such by present methods.

PURE CULTURE BY SEMI-MECHANICAL METHODS

Pure culture by semi-mechanical methods places emphasis on the isolation instruments or isolators employed, as was detailed in the previous report (Hildebrand, 1938). Most if not all of the reports published during the past decade are modifications of the older published methods, ordinarily involving the spreading of spore suspensions on an agar surface and the use of a tool fastened somehow to the microscope to separate an isolated cell for transfer to a sterile medium. Several of the more important reports will now be briefly discussed.

DRY NEEDLE METHOD. Chilton and Wernham (1940) mounted a simple single-spore isolator needle on the condenser holder of a microscope for manipulating in an isolation chamber consisting of a Van Tieghem cell, glued to the center of a piece of wood the size of an ordinary microscope slide with a hole, of the same diameter as the inside of the cell, in the bottom. The point of the needle was centered in the microscope field by manipulating the arm holding it.

Martin (1943) employed a simple technique for isolating spores of various fungi from exposed slides in aerobiological work. First, under the low power of the microscope the location of a spore or group of spores was marked with a crow-quill pen and India ink. Second, the slide was then inverted over a Van Tieghem cell, and single spores were isolated by means of a micromanipulator, according to Hanna's Method (1928). Third, if spores germinated and produced sporulating cultures, they could be identified by their manner and kind of spore formation.

Tapke (1946) devised a rapid method for isolating single ascospores from apothecia. Application of alcohol to the outer and lower surfaces of apothecia caused instantaneous ejection of ascospores of various species of *Sclerotinia*, a method slightly modi-

fied from that taught him by the late H. H. Whetzel in 1917. Three Petri dishes containing nutrient agar were placed near the vial containing the apothecia. These dishes should preferably exceed the fruiting body only slightly in height and diameter and be opened in the spore cloud in quick succession. The last plate usually catches only a few spores in perfect position for isolation and quite free from contaminants. Very small apothecia were held face downward over the nutrient agar and rubbed with a needle moistened with alcohol to cause ascospore ejection.

CYLINDER LOOP NEEDLE METHOD. Lambert (1939) announced a spore isolator combining some of the advantages of the Keitt (1915) and La Rue (1920) methods. The instrument is essentially a "biscuit cutter" mounted vertically in a threaded brass plug, screwed on the microscope in place of the oil-immersion objective. In practice it was found advisable to make the "cutter" about ¾ mm. in diameter or slightly less than the microscope field under the low power. The cutter can be centered by bending the wire so that it will mark off a circle concentric with the low-power field of the microscope. While possessing the mechanical precision inherent in the La Rue (1920) method, it also is adapted for lifting out the agar disc bearing the spore so that transfer can be made with no danger of the "spatula" touching the surrounding agar.

McLean (1939) described two improved types of cutter plunger, attachable to the nose-piece of a microscope, for cutting out portions of agar bearing single spores. One is fitted with a safety spring, and both are supplied with a closely fitting ramrod for pushing out the agar by air compression.

Georg (1947) reported a simple and rapid method for obtaining monospore cultures of fungi. For isolating spores 3 μ or larger, a small conical glass tip was made from a length of glass tubing with the larger end fitting round the lens of the low power objective (6 to 7 mm.) and the smaller end approximating the microscopic field as seen through the lens (1.5 to 1.8 mm.). The glass tube was heated to redness and drawn out to the required shape. A thin ring of plasticine was fitted round the lens of the low power objective, and to this the sterile conical glass tip was affixed. When the spore is centered in the microscopic field, the objective can be lowered so that the small end of the tip cuts a

disk of agar containing the spore. The disk is then removed and transferred by a sterilized needle.

Northcraft (1948) described and illustrated a rapid method of single cell isolation involving an isolator fastened to the microscope condenser. A piece of sterile 4.0 percent agar, about 10 mm. square and 0.5 mm. thick, was placed in the center of a 22×40 mm. coverslip. The cells to be isolated were gently streaked with an inoculation needle onto the surface of the agar square. The coverslip with the inoculated agar square was then inverted over and centered in the window of the Lucite slide, a drop of glycerine at each end of the coverslip anchoring it to the slide. The mount is placed on the microscope stage and examined under the low (about $100 \times$) power. Each cell can be studied under high power. When a cell is selected, the condenser is raised until the glass tube shadow shows. After centering, the cells are ringed by raising and lowering the condenser. The slide is then placed agar side up under a binocular dissecting microscope where cell and agar plugs are removed by a microscalpel for transfer to a test tube culture medium.

INDIA INK DROPLET METHOD. The India ink method, discovered by Ehrenberg (1838), is fully reviewed by Niklitschek (1939). The ink may be rubbed up with distilled water in a depression of a white porcelain plate, using a clear glass rod. Addition of ink should stop when the color of the ink creeping up the margin changes from brown to jet black. Trituration, however, should continue for at least 15 minutes longer. A "dry method" is employed for isolating blood parasites with a drop of blood mixed with a drop of India ink. The so-called "wet method" was used for isolating protozoa by employing equal amounts of culture fluid and India ink in mixture.

MICROCOLONY METHOD. De Zeeuw (1943) produced a special nutrient medium to obtain single-spore cultures of *Agaricus campestris*. A large number of uncontaminated spores were smeared on an agar slant with a sterile brush and allowed to germinate and grow for one to two weeks. After a solid mycelial mat had formed, the culture was killed by immersing the tube in boiling water until the medium was completely liquid. The dead mycelial mat was then removed aseptically and the

sterile medium used for hanging drops on cover slips over Van Tieghem cells. Instead of only five percent germination by the usual methods, from 30 to 50 percent of the single spores of two commercial varieties of mushrooms isolated and placed on this medium germinated in replicate tests.

MISCELLANEOUS METHODS. Harding (1939) developed a simple instrument for dissecting minute animal organisms. It is based on the principle of the pantograph and is attached to the microscope for focusing without using the hands. With it one can make a permanent mount under one coverslip of all the appendages dissected from one small crustacean.

Lloyd (1940) designed an object-marker for the microscope which occupies one of the apertures in the triple nosepiece. When centered, the marker is brought down by the focusing adjustment into contact with the coverglass. Then the marker is given one complete rotation, causing the point to describe a circle whose diameter depends upon the adjustment previously made. A selected splinter of carborundum makes a neat circle which lasts for a considerable time.

Olson (1942) describes a device for marking fields on microscope slides. It consists of removable tips fitted into the nosepiece of the microscope which when centered to the area and inked can be lowered until the end of the tube touches, leaving a ring of ink around the field to be marked.

Hegre and Blount (1945) have perfected a chin-operated focus adjustment to free the hands when both are required for the manipulation of the object under the dissecting microscope.

MICRONEEDLE AND MICROPIPETTE INOCULATORS. Storey's (1933, 1938, 1939) series of micrurgical investigations is the most outstanding that has been conducted on insect transmission of plant viruses. In 1933 he illustrated his methods of inoculation by means of a special microneedle and micropipette, using semi-micrurgical methods—performing the operations under the dissecting microscope. The reader is referred to these papers for further details.

Black (1941) transmitted the aster yellows virus mechanically by micropipette from insect to insect, that is, the diluted juice extract from the bodies of viruliferous leafhoppers held at 0° C.,

when injected into the insect abdomens at dilutions as high as 1–100 in 0.85 percent NaCl solution, was effective in transmitting the virus to healthy leafhoppers. This feat has thus far not been accomplished from diseased to healthy plants by mechanical means.

White and Braun (1941) demonstrated crown gall (plant cancer) perpetuation by bacteria-free tumor tissues. For initiation of galls in the first place the host cells must be under the influence of the crown gall bacteria (*Agrobacterium tumefaciens*) until they acquire the capacity for autonomous growth. Tissue fragments from the occasional crown gall tumors on sunflower stems were successfully cultured *in vitro* on special nutrient media and shown to be bacteria-free. When implanted by micrografting into healthy plant tissue, some typical crown gall tumors developed.

Blakeslee (1944) described a method whereby small embryos from artificial cultures or from mature small seeds may be grafted onto normal stock of *Datura stramonium* with protection against evaporation by means of gelatin capsules. By this method of micrografting, the excised embryos, which fail to develop roots, developed into useful seedlings.

PURE CULTURE BY MECHANICAL METHODS

Several new instruments have been added to further extend the extreme diversity in micromanipulator design. Chambers (1940) reviewed recent developments of the micromanipulative technique and its applications. His article is a shortened and somewhat modified account of a chapter prepared by him and M. J. Kopac for McClung's (1937) "Handbook of Microscopic Technique".

Micromanipulators

PNEUMATIC PRINCIPLE. At the time the first report was prepared (Hildebrand, 1938) the writer had no definite citations on the new micromanipulator which was being developed by De Fonbrune in France. First note to attract attention was made at the University of Montreal by Brunel (1938) who mentioned the De Fonbrune pneumatic micromanipulator in reviewing recent progress in the science of micrurgy. Several important articles have been published by De Fonbrune (1932, 1935, 1937). In the latest article (De Fonbrune, 1937) the new pneumatic micro-

manipulator, the receptor (isolator holder) and the microforge are described along with the use of the latter for the preparation of microinstruments. In Part 1 a detailed description is given of the pneumatic micromanipulator and how it functions for the operation of the receptor which holds the micro-instruments. Part 2 deals with microforge and its makeup. Part 3 gives the techniques in the use of the microforge for preparing a wide variety of microinstruments. The price quoted then for one micromanipulator, one receptor and one microforge was between 10,000 and 15,000 francs.

SIMPLE LEVER PRINCIPLE. Buchthal and Persson (1936) devised a micromanipulative apparatus which permits accurate work at magnifications up to 500 diameters. This micromanipulator is similar to the Emerson (1932) apparatus but operates with several concentric ball-and-socket joints with one lever only. It uses the screw-and-slide principle for gross movements. In operation a lever turns a ball. The length of the lever can be varied with the length of the lever ratio to the offset from the ball, determining the reduction in the movements performed.

MISCELLANEOUS DESIGN. In 1938 Hanson (1938) announced a new design of micromanipulator which was capable of allowing motion of the isolator (quartz needle or other fine tool) for small distances and in any direction. This instrument is of multiple design. According to the report the conditions attained are: a considerable ratio between the motion of the operator's hand and the needle, good rigidity, freedom from backlash, freedom from wear (a defect of the Barber (1904, 1914) instrument) and ease of construction. In a test for lost motion it was found that the average difference in micrometer settings amounts to about one wave-length of light.

SCREW-AGAINST-SPRING PRINCIPLE. Pyke (1939) devised a simple micromanipulator mounted on a rigid base plate. The base plate consists of angle steel measuring three inches by three inches by ¾ inch and seven inches long. The movement of the needle or pipette is controlled by two rigid T-shaped levers, A and B, the needle being attached to lever B. The fulcrums on which these levers rock are formed by steel balls ⅛ inch in diameter which engage in grooves filed on the vertical members.

HYDRAULIC PRINCIPLE. Cailloux (1943) described a new hydraulic micromanipulator composed of two units, micromanipulator and receptor, which holds the microtools. The manipulator is essentially composed of a lever mounted on a universal joint to which are attached three pistons of small diameter. When the lever is moved by the operator, the liquid is displaced by the pistons in each of the three tubes connected to the receptor. The liquid finally acts upon each of the three flexible diaphragms on the receptor connected to the main shaft bearing the microtool. The pistons are not coupled in rectangular coordinates. Instead, the connecting rods are all attached to the same point on the main lever and, with the pistons, seem to form the edges of a pyramid. Thus in a straight horizontal movement, the three pistons displace equal amounts of liquid in the tubes. The membranes of the receptor are connected similarly. This method of grouping the pistons and membranes simplifies the mechanism of the instrument and leaves a minimum of articulations, thus avoiding loss of movement through play between parts.

The Cailloux hydraulic micromanipulator offers several advantages: (*a*) all movements of the microtools are controlled by a single knob; (*b*) positive response without lag of the microtools; (*c*) parasite vibrations of the manipulator are not transmittted to the microtools; (*d*) to obviate inversion of the image in a microscope, the receptor can easily be placed in such a way that the microtool seems to be the continuation of the lever on the manipulator; (*e*) the ratio of demultiplication can be varied at will; (*f*) it is suggested that the instrument user does not need previous training to use it expertly; (*g*) in its manufacture very few parts need to be machined with great precision.

Of the new instruments described, the writer has tested and operated only the Cailloux instrument located at the University of Montreal.

Isolators

Several designs of isolators, or instruments used in micromanipulative technique, have been used in the past. Some special machines have been invented for fabricating microinstruments, of which the L. G. Livingston "pipette puller" is an outstanding example. Undoubtedly more progress has been made in the past

decade in the invention of instruments for the fabrication of microtools than in the microtools themselves.

MACHINES FOR FABRICATING MICROINSTRUMENTS. De Fonbrune (1937) describes in detail his new instrument, the microforge. After giving a detailed description of the pneumatic micromanipulator in Part 1, Part 2 deals with the microforge and its makeup, and Part 3 gives the techniques in the use of the microforge for preparing a wide variety of microinstruments, including microrods, microelectrodes, microloops, microscalpels and micropipettes.

Comandon and De Fonbrune (1938) made a study comprising four parts: (*a*) general technique in the preparation of the moist chamber; (*b*) the physical conditions—optical quality, capillary action, aseptic condition and moisture—in the chamber; (*c*) special techniques—fixation and coloration of the preparations, quantities of material, microcultures on gelatin or cellophane, and tissue culture; (*d*) using the chamber in micromanipulations for isolation of microorganisms.

Thaysen and Morris (1947) describe their extensive preparation of microtools for use with the micromanipulator. Because the tools required for the more delicate processes of micromanipulation are not commercially available, and existing equipment for their making is expensive, some cheaper equipment is described, comprising a gas forge and an electric forge, which together suffice to prepare the tools needed for isolating single cells, for injecting and dissecting living cells and tissues, and for determining their electrical potential. The microtools illustrated are microloop, microneedle, micropipette, microelectrode, microknife and microburner.

MICROINSTRUMENTS. Microinstruments when motivated by a mechanical hand (micromanipulator) can function in many ways, depending on the design of the instrument. While many microtools are made by hand, special machines have been designed for pulling or drawing micropipettes.

Richter (1947) invented a precision micropipette trimmer for perfecting the tips of micropipettes and microneedles for use with the Chambers' micromanipulator.

Hofmeister (1940) used the Schouten (1935) and Leitz-Du Bois

(1931) pipette pullers. For his micromanipulative work on diatoms he used a so-called Gleitmikromanipulator von Zeiss and the Chambers micromanipulator. He assembled a double instrument, employing two microneedles in two holders (left side, micropipette; right side, single microneedle) for micrurgical studies. For microinjection studies involving plant cells he again employed both micromanipulators. He studied onion scale slices and injected vacuoles with one-micron pipettes, using water as a head instead of oil. Cells were also injected with other chemicals and substances including sugar and calcium chloride.

Sheffield (1939) performed numerous microdissection, microinjection and microinoculation studies with plant viruses. She tested the cell contents, the stability of intracellular inclusions, the virus content of the cells. By means of a microcutting needle device, cells were divided into two parts with one containing the inclusion body. The inclusion body was then removed by exerting very gentle pressure at the base of the cell or by replacing the microcutting tool with a micropipette and removing the body by suction. Usually ten to twenty inclusion bodies were collected before diluting same for inoculation purposes.

Kassanis and Sheffield (1941) in their micrurgical studies reported variations in cytoplasmic inclusions induced by three strains of tobacco mosaic virus: spindle-shaped bodies, masses of short, needle-like fibers, extremely long coiled fibrous forms, and new amorphous forms. All these arose either directly or from preexisting inclusions of the previously recorded types.

Hildebrand (1941) in micrurgical studies demonstrated that more than 90 percent of crown gall bacteria (*Agrobacterium tumefaciens*) grew in sterile juice extract from young tomato plants. Single bacterial cells microinoculated into deep wounds on tomato stems produced galls in ten to 60 percent of the trials compared to ten percent or less in shallow wounds. When ten or more bacterial cells were injected, gall formation resulted in about 90 percent of the trials with the deep wounds compared to 20 percent or less with the shallow wounds. Thus it appears that depth or type of wound is more important than number of bacteria. Individual plant cells when injected with one or more bacterial cells always gave negative results. Living bacteria were recovered from only one of 20 plant cells as late as three weeks after

inoculation. Minute wounds, produced by gently rubbing a polished needle with bacteria on stems and petioles, resulted in tiny galls close to the minute wounds associated with collapsed hair cells. Very few bacteria were observed in the tiny galls. A further elaboration of this study appeared the following year (Hildebrand, 1942).

Schure (1934) reported damaging action of substances dissolved from glass in single-cell cultures. This discovery was made in an effort to obtain pure cultures of *Reticularia lycoperdon*. The damaging substance suspected was probably silica.

In 1929 Whitaker (1929) described the construction of microthermocouples, small enough and strong enough to be inserted into small living cells or tissues. They are small enough to measure light absorption of a single plastid in a cell. The method is to draw various metals in glass to wires or filaments of exceedingly small size to make electrodes of less than one micron in diameter. Iron and platinum were found to work better than copper and constantan.

Burch (1939) revised a micrometer screw top fitted to the plunger of a tuberculin syringe so that small movements can be made with accuracy. For calibration the microsyringe is filled with mercury and the amounts ejected are weighed.

The Dean and Fletcher (1942) micrometer burette combines a micrometer with a syringe. The anvil of the micrometer is cut off and a glass syringe mounted on a simple clamp in line with the spindle. This instrument can be mounted and held by a universal burette clamp which can be attached to a ring stand. The micrometer burette can be calibrated to one part in 1000 independent of the speed of delivery. It can be dismounted and cleaned and oven-sterilized if necessary, permitting use of several solutions in the same burette. This would seem to be a superior instrument to the microburette recently described by Scholander (1942).

The Scholander (1942) microburette is a modification of the Rehberg (1925) burette. In it mercury is displaced by the spindle of a micrometerlike device. Moving an ordinary micrometer spindle through 25 mm. displaces around ¾ cc., which is precisely divided into 2500 parts with an accuracy of 0.1 mm. The solution is sucked into the bulb by turning the spindle, and

the instrument is ready to use. The disadvantages of employing mercury are that the instrument gets dirty easily and can be used only with solutions that do not attack mercury.

Conger (1944) developed a simple method for micrometric ruling, and some applications. A crystal of silicon-carbide or carborundum, or a black diamond is mounted with sealing wax or dental impression compound on a micromanipulator or other rigid lever which is regulated by two sets of screws. This is attached to the front of the microscope with the crystal in the field of vision and serves as a cutting tool. The mechanical stage forms the ruling engine. As many as 30,000 lines per inch can be made without difficulty.

Wagner (1945) has developed an experimental microdrill for obtaining material for a microchemical test from some of the harder minerals. It consists of a motor mounted on a wooden base, a flexible shaft connected to the motor at one end and to a handpiece and chuck-drive arrangement at the other, and a chuck to hold the cutting tool.

The Arens (1939) apparatus determines the turgor pressure in a single cell with a manometer and has been used for measuring turgor pressure of large cells of *Nitella, Caulerpa* and *Valonia* without injury to the cell. All that is necessary is to place a light behind the window of the apparatus and project the bending of the cell on a screen, while the pressure can be read on an illuminated manometer.

Policard (1939) reported on a microincineration method for studies on mineral distribution in cells and tissues. If properly performed it gives a reasonably true representation of mineral distribution in living cells and organisms.

Schaede (1940) demonstrated root nodule bacteria in living gall cells.

Castle (1940) measured the rotary component of growth of a single plant cell, the sporangiophore of Phycomycetes, and observed significant differences in the increments in growth in successive 1/5 second intervals with the average change in growth rate between two and five percent of the average.

Chambers (1941) demonstrated that the so-called "blue nuclei" in tulip petals are the result of injury causing the pigment, which

normally is restricted to the cell vacuole, to be preferentially absorbed by the nucleus when released into the cytoplasm by crushing injury.

Chambers and Black (1941) demonstrated the appearance of large numbers of phantom nuclei upon deplasmolysis of cells previously exposed to electrolytes, whereas vitreous nuclei appeared infrequently.

Lotze and Yiengot (1941) devised an apparatus for measuring microscopic objects, such as the marginal bodies occurring in the erythrocytes of infected cattle. The apparatus measures down to tenths of microns and consists of a microscope fitted with an oil-immersion lens, a 15 × ocular, and a camera lucida from which the mirror had been removed plus a light box two feet square and one foot deep fitted with four electric lights and placed about ten feet away from the microscope in a lateral direction. The open side of the box was turned toward the opening in the side of the camera lucida and was covered with a cardboard in which the scale had been made.

French and Anson (1941) demonstrated oxygen production by isolated illuminated chloroplasts suspended without much other cellular matter in sucrose solution with buffer, ferric oxalate and potassium ferricyanide. Spinach proved a good source of active chloroplasts. The reaction appears to involve catalysis by an enzyme, since it is stopped by warming, by Duponol, a protein denaturant, and by enzyme poisons. At low temperatures more O_2 is obtained, due to the high temperature coefficient (3.0) of inactivation of some constituent. The activity of a certain preparation of chloroplasts suspended in 0.5 M. sucrose had a half-life of 26 minutes at 35° C. An improved method of preparation of chloroplasts was carried out at a low temperature. Chloroplasts may be stored for a day or two and lose activity much more slowly in nitrogen than in air.

Gordon and Chambers (1941) studied the relation of particle size of a number of water-soluble acid dyes to their penetration into three types of living animal cells (tissues). Dyes with particle radii of 5.5 ± 0.4 to 6.4 ± 0.5 Å or less penetrated the ciliated epithelial cells. The upper limit for penetration of muscle fibers or bladder epithelium was 6.5 ± 0.4 to 7.3 ± 0.9 Å.

Seifriz and Epstein (1941) demonstrated shock anesthesia to protoplasm in their experiments employing one of the myxomycetes, *Physarum polycephalum*.

Guttenberg (1943) isolated plant cells of the stigma mucus of *Cymbidium lowianum* and cultured them in an attempt to secure evidence for the existence of hormones for cell proliferation. The substances tested (heteroauxin, thiamin, etc.) markedly stimulated the elongation of the cells, but cell division was not observed in any case. The enlargement induced was not typical of the proliferation process, and accordingly no specific substance has been tested which promotes cell proliferation. This report neglects to mention the wound hormone traumatin.

Moist Chambers and Special Chambers

Comandon and De Fonbrune (1938) devised a special moist chamber and discussed the advantages from its use for the study of living microorganisms, tissue cultures, and in micromanipulation, including single cell isolation, microdissection and microinjection.

Thaysen and Morris (1947) describe a simple hanging-drop micromanipulation chamber. The chamber was designed to accommodate the various microtools needed for isolating single cells, for injecting and dissecting living cells and tissue, and for determining their electric potential.

Kamiya (1940) placed small bits of *Physarum polycephalum* plasmodia on cover-glasses coated with agar and noted that they soon spread into thin sheets which later developed protoplasmic strands. One form developed that could be arranged so that there were two protoplasmic bodies connected by a single strand. Such a plasmodium was inverted over a chamber which was divided into two compartments, A and B, by an agar block. The construction is such that the two compartments may be kept air-tight without the wall separating them blocking protoplasmic flow in the connecting strand. One of the two compartments is kept at constant atmospheric pressure, whereas the pressure in the other compartment is under control. Protoplasmic movement goes on normally when pressure is same. Difference in pressure alters flow—reduced pressure accelerates flow; increased pressure retards flow. Removal of pressure causes normal flow again.

Thomas (1941) described a technique for continuous microscopic observation. The cavity of a depression slide was filled with an excess of freshly heated (sterile) oil. A small portion of a semi-liquid culture of the organism to be studied was then transferred by pipette from bottom of culture tube to the center of a sterilized coverslip. The coverslip is inverted over the depression of the objective slide in a manner that will eliminate all bubbles of air. The excess oil will be displaced to form a perfect seal. The microscope should not be disturbed because in a prolonged observation of a specific germinating spore or a dividing cell, any movement of the microscope may cause the organism to be carried from the field. By changing the distance between bulb and stage or by varying the intensity of bulbs, one can obtain the temperatures desired.

Brown (1942) developed a method for the study of the development of fungi (*Aspergillus niger* and *Monilia* spp.) from single spores or hyphal fragments. It involves a special glass slide ($76 \times 28 \times 2$ mm.), the center of which is occupied by a polished, slightly depressed, concave table chamber, 1 cm. in diameter, surrounded by two concentric moats connected by a short communicating moat and providing for an air inlet. A square 25 mm. cover glass is held in place by mineral oil contacting six points on the slide after a full, large (4 mm.) loop of inoculated culture medium (Sabouraud's agar) has been placed at the top of the central table. Contamination and desiccation are obviated by restricting air to that entering through the moats out of contact with the culture medium.

Weston (1942) made a Petri dish holder for mechanical stages which firmly clasps the dish and carries it by a frame that fits snugly into the slide holder of the mechanical stage. A further modification was a device for holding the Petri dish top over the bottom to permit mechanical manipulation of needles and pipettes in the dish.

Horner (1945) designed three stage incubators to be used in microscopic study. These instruments afford the study of precisely predetermined volumes of culture media under the microscope, with closely controlled conditions affecting development of the culture in respect to variable temperature, variable electromagnetic or electrostatic field, or a combination of these or differ-

ent conditions. It is often desirable to be able to determine accurately the rate of propagation. This may be accomplished by accurate counting from time to time of the number of cells present in a precisely determined volume of the medium. It is desirable to have quantities sufficiently small for counting directly, or by dilution, in a known volume, and to permit counting of subsequent divisions of the cells in a given fraction of the original volume. The stage incubators are constructed of a non-corrosive material which may readily be cleansed and sterilized at high temperature with or without the use of a disinfectant.

Richter (1948) invented an improved moist chamber slide for use in micromanipulation. The method of keeping the chamber moist precludes chance disarrangement of manipulation needles while in use and provides a measurable control of relative humidity.

Light Source or Mechanism

Undoubtedly the finest lamp for micrurgical studies is the new so-called "Universal lamp" put out by Spencer Lens Division of the American Optical Co. at Buffalo, New York. By means of a transformer and rheostat the light intensity can be varied at will. A distinctive feature is that the lamp does not heat up and therefore has no operation hazard from burning on contact. Because of the low and steady heat component in its light rays, one can operate the instrument for one or two hours without the condensation droplets in the moist chamber being destroyed by enlargement and running together.

Melezer and Venkel-Wlassics (1939) suggest the use of a quicksilver vapor lamp as a light source for fluorescence microscopy and microphotography. Their lamp has a wave length between 310 and 390 millimicrons and operates on 220 volts A.C. This lamp was developed by the Simons Company and is suggested for use in place of the expensive low-voltage ultraviolet arc lamp using iron electrodes. This lamp is designed on the general plan of the ordinary tungsten incandescent lamp with Edison base, and burns evenly, emitting a constant source of light.

THE BURCH REFLECTING MICROSCOPE

The birth of the science of bacteriology was made possible by the invention of the light microscope. This instrument, however,

even after being perfected, has limitations, and the recent invention of the super- or electron microscope, while assisting in the problem, has not completely filled the long felt needs within the range of light microscopic objects. The invention of the reflecting microscope by Burch (1947?) certainly promises a significant advance in this direction.

While the idea of microscopes using mirrors, similar to those of astronomers, instead of lenses is not new, the most significant development along this line is the Burch reflecting microscope, as discussed by Barer (1947). Barer states that its two unique features are that it is completely achromatic and that it has an unusually long working distance. It is ideally adapted for absorption spectroscopy of cellular constituents. Whereas the quartz microscope is suitable only for plotting an absorption curve over a very narrow band of wave lengths, with the reflecting microscope the entire absorption from the far ultraviolet to the infrared can be plotted on the one instrument. The quartz microscope cannot be used below 1900 Å, since quartz becomes opaque in this region. The fluorite lenses could be used down to about 1300 Å, but fluorite is very expensive and such lenses are difficult to make. Below 1300 Å no substances suitable for making lenses are known. It seems reasonable to suppose that the reflecting microscope could be used down to 1000 Å, at least, and probably even lower. If the immense difficulties can be surmounted one day it may be possible to use wave lengths approaching 100 Å, and this would provide an instrument with a resolving power of the same order as the present electron microscope.

In the present reflecting microscope, when in focus the distance between the mounting of the small mirror and the object under observation is 13 mm. This distance could be increased to 20 mm. if the mountings were redesigned. For various types of micrurgical work, and especially for micromanipulation, long working distances are a great advantage particularly because they enable a microinstrument to be manipulated between the cells observed and the small mirror.

According to Barer (1947), ultraviolet microscopy should be extremely important in biology for two reasons. In the first place, the shorter the wave length of light, the higher the resolving power and useful magnification of the instrument. Thus

ultraviolet light of wave length 2500 Å should give about twice the magnification obtained with green light of wave length 5460 Å. Secondly, certain constituents of the living cell selectively absorb some of the ultraviolet wave lengths with the cell nucleus showing a strong absorption in the region of 2500–2700 Å. Unstained living cells photographed in light of such wave length will therefore appear as if the nuclei have been stained. Here we have the great advantage of the reflecting microscope. To take a photograph by ultraviolet light, one focuses the image by visible light, changes to the ultraviolet source, and takes a photograph without change of focus. The same thing also holds for infrared light. Thus ultraviolet light of any wave length can be used with the reflecting microscope, which is not true for the quartz microscope, essentially a monochromatic instrument, that corrects for only one wave length.

Barnard (1939–1940) studied the value of ultraviolet microscopy for the investigation of the smallest living things—small bacteria and viruses. He clearly delineates the fact that the ultraviolet light technique—observing a visible dark-ground image and determining the size of observed bodies in terms of their brightness—is apparently little appreciated by microscopists. The same method is well known and widely used in astronomy. The ultraviolet light technique was used for photographing eight species of bacteria at magnifications between $\times$ 2000 and $\times$ 3200, and seven species of animal virus all at the $\times$ 2250 magnification. Some of the viruses appeared similar to some of the larger recognized microorganisms. None of the viruses appeared similar to ordinary bacilli. The resemblance of the viruses to cocci was much closer. The vaccinia virus departs from the spherical shape only at subdivision, behaving at that time like micrococci. When the ultraviolet light print showed dense opaque masses, the virus particles apparently had a size of less that 50 $\mu\mu$.

Micrurgical science should benefit tremendously when more of these instruments become available.

GERM-FREE CULTURE OF ANIMALS

Glaser and Coria (1935) described a simple method for preparing yeast extracts useful for culturing certain protozoa. *Paramecium multimicronucleatum* was purified and cultured on a

special medium. Cultural differences between these species and *P. caudatum* were noted. Three ciliate strains from thermal waters were also purified and cultivated. This work demonstrated that protozoa could be freed from other living microorganisms and that they yielded biological reactions that may prove useful in the separation of morphologically similar forms.

Glaser (1943) points out that in the germ-free culture of invertebrates it will be necessary to recognize that in nature, after species identification and genetic purifications, every animal is still a combined zoological and botanical garden. By special reliable techniques he was able to grow certain invertebrate animals (protozoa, nematodes, insects) germ-free. Can one imagine a cultural growth in a test tube containing millions of teeming nematodes in pure culture?

Hoepple, Feng and Chu (1938) attempted to culture nine species of helminths of vertebrates in artificial media. Isotonic salt solutions gave the best results for intestinal helminths. Serum, undiluted or diluted with or without various substances, gave good results for bile-duct, blood and tissue parasites. Transfer to fresh media at intervals is necessary for keeping parasitic stages alive over a long period. The longest survival period was about five months for *Chlorchis sinensis.*

Johnson and Baker (1942) developed a successful culture medium for growing *Paramecium multimicronucleata* germ-free, employing pressed yeast juice sterilized by passage through a Seitz bacteriological filter.

Jackson (1945) developed a method for observing and staining live insect blood under oil immersion. The procedure consists essentially of immobilizing a larva beneath a coverglass in such a position that one antenna is pressed against the glass, cutting off the end of the antenna, and observing the blood as it pulses forth against the glass.

SINGLE CELL ISOLATION BY THE TREXLER AGAR-COVERSLIP METHOD[3]

1. Apparatus. Reyniers (1938) micromanipulator and moist chamber; special agar freed from debris; ring slides; coverslips

[3] The method here described was demonstrated by Philip C. Trexler on November 14, 1940, before the Advanced Plant Pathology 201 class at Cornell University, Ithaca, New York.

(¼" square and 0 thickness); shellac, paraffin, porcelain, vaseline; microneedles; micropipettes.

2. Clearing of agar. Autoclave Difco Brain Heart Infusion agar (1.5%) in centrifuge tubes of about 15 cc. capacity. After sterilization the tubes are placed in a heated metal cup with a water cushion. Balance and heat the cups with the tubed agar to about the boiling point. Centrifuge at high speed for about 15 minutes until agar is solidified. Hold the tube in a strong cross light; if it looks clear it should be satisfactory for use. Other methods of clearing agar (centrifuging, white of egg, etc.) can be used instead. From experience he found that by heating in the Arnold steamer, most particles usually settle out in about one-half hour.

3. Preparation of ring slides. Materials tested include varnish, paraffin, porcelain cement and brass rings. Porcelain cement has definite advantages. Insolute porcelain cement comes in bottles in powder or paste form. With a toothpick or other device several porcelain (or paraffin, etc.) ring slides are prepared to act as chambers for holding the coverslips. The ring on the "ring" slides should be about 1/32 inch high and somewhat less in diameter than the coverslip. More recently brass rings have replaced all the rest because it is easy to keep their surface parallel to the slide. Trexler preferred ring slides to depression slides.

4. Preparation of Coverslips.

(*a*) Check "ring" slides. If uneven, polish surface smooth with a stone. Examine for depth and diameter correctness.

(*b*) Prepare a bulb pipette, using a 1 cc. rubber bulb and a piece of glass tubing drawn out to a fine tip.

(*c*) Clean the coverslip (Preference is for ¼ inch square glass of No. 0 thickness) by dipping in alcohol or acid-alcohol followed by alcohol. Then polish with lens paper. Hold coverslip in a pair of forceps.

(*d*) Carefully heat a tube of agar over a Bunsen flame. Apply the heat near the top of the agar column only, so that perhaps the upper half inch becomes melted.

(*e*) Flame the coverslip in order to sterilize and heat it above the temperature of agar solidification.

(*f*) Likewise flame the pipette for the same purpose.

(*g*) Suck up about ½ cc. agar in the pipette and place a drop on the warm coverslip. Turn the coverslip at an angle so that the

fluid flows to one side of the drop and remove as much as possible with the pipette. Immediately set the coverslip on the ring slide. Press firmly on the vaseline seal so that a tight connection is made.

5. Procedure.

(*a*) A coverslip is inoculated with a young culture of the organism being studied.

(*b*) The inoculated coverslip is placed on the moist chamber located on the microscope platform.

(*c*) Single cells are isolated by the Dickinson (1926) method.

(*d*) The locations of from six to 12 well isolated cells are marked on each coverslip by tearing the agar film with the microneedle.

(*e*) The agar coverslip is then transferred to the "ring" slide for incubation.

(*f*) When microcolonies develop, the agar coverslip is replaced on the moist chamber.

(*g*) Microcolonies are transferred to culture.

6. The Trexler modification of the Reyniers (1932) micropipette puller.

(*a*) The principal modification was to pull micropipettes with a bent tip.

7. A Tungsten (Pfansteel) microneedle for use in micrurgy. The idea is to polish a tungsten wire to a very fine point by electrolysis, using the principle of "atomic polishing".

(*a*) A battery jar arrangement is set up with the jar containing 1 M. NaOH and a reducing agent such as sodium hyposulfite.

(*b*) Two electrodes of tungsten were installed in the improvised cell and held by battery clamps.

(*c*) A transformer (15 volts or less) is connected. Either A.C. or D.C. may be used.

(*d*) If the wires are dirty they can be cleaned in potassium dichromate solution.

(*e*) When employing a 15-volt transformer, a fine tungsten tip is formed in about two minutes.

(*f*) The depth of the tungsten wire in the NaOH solution and the amount of metal removed determine the taper.

(*g*) The tungsten microneedle can be used for many purposes, especially for transfer of microcolonies. The great advantage of this microneedle is that it can be flame sterilized and used again and again.

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GROWTH OF FUNGI IN SYNTHETIC NUTRIENT SOLUTIONS. II.[1]

ROBERT A. STEINBERG

U. S. Bureau of Plant Industry, Soils and Agricultural Engineering, Beltsville, Md.

INTRODUCTION

The years that have passed since publication of the previous review (108) have covered a period of expanded interest in the nutrition of fungi. The previous investigations on the organic and mineral needs of fungi had laid a firm foundation for these more recent studies. Investigations were barely under way at that time on the genetic factors concerned in the erratic behavior and "degeneration" of fungi (mutation) in culture. A fresh impetus too was given by studies of antibiotics and fungal decay of materials, since little was known of the specific nutritional requirements of the fungi dealt with, or of their capabilities.

While it is true, therefore, that progress has been made, it is also a fact that a great deal still remains to be done. Some fungi, such as the rusts, the powdery and the downy mildews, cannot be cultured on any media. Other important problems awaiting solution are, to mention but a few, the relation between constitution and assimilability of the hexoses and the chemical reactions of their transformation into amino acids. Though fungi offer excellent material for studies on mineral deficiencies at fixed carbohydrate levels, no investigators have concerned themselves with their relation to protein-carbohydrate metabolism. The importance of resistance and pathogenicity in fungous diseases has not as yet led to any intensive studies of the products of fungal exosmosis in culture from this viewpoint. These products probably play an important part in these phenomena.

Reviews of various phases of investigation bearing on nutritional studies have appeared from time to time during the interim. Schopfer (106) and more recently Robbins and Kavanagh (90) have reviewed what is known on the vitamin requirements of fungi. Perlman (84) has summarized the data on micronutrient requirements, though primarily with relation to the products of

[1] Supplement to article in The Botanical Review **5**: 327–350. 1939.

fermentation and not growth. An excellent summary on mutations and variations in fungi pathogenic to plants has been published by Christensen, Stakman and Christensen (19) and in *Neurospora* by Beadle (4). Microbial genetics has also been reviewed by Lindegren (64).

The subject matter of this review includes the nutritional requirements of fungi for carbon and nitrogen compounds, minerals and vitamins for growth and reproduction. A brief section on mutation in fungi has also been included as in the previous review and for the same reason—a variable organism cannot be used to obtain reliable and constant results in nutritional or pathological studies. The short section on pathogenicity has been added in the hope that it may induce investigations in synthetic solution of the products of exosmosis of fungi, and of the influence of nutrition thereon.

This review makes no pretense at completeness of coverage. Many probably important articles could not be obtained, whereas others were in languages unfamiliar to the writer. In an attempt to conserve space, the names of the fungi are given without mention of taxonomic authorities, even though stated by the author. The citations, lastly, are not mentioned in chronological order of publication but usually in an alphabetical arrangement according to the author's name.

CARBON REQUIREMENTS

Additional information on carbon utilization is largely confined to the extension of previous results to additional species. The data are usually on the basis of compound percentage content. Comparative results on carbon utilization would be of still greater value if equal carbon or energy levels, or molecular weights were used. An insight might so be had into the chemical factors concerned with carbon metabolism. It is also important to remember that results with mixtures of carbon compounds are not directly comparable to those with single compounds. Mixtures may be metabolized more readily in some instances than are single compounds. Or a compound practically unusable may be readily employed on admixture with another.

Most fungi grow readily with sucrose and the hexoses—D-glucose, D-mannose, D-fructose—, and in addition the pentoses

D-xylose and D-arabinose to a lesser degree. A comparative study of growth of *Aspergillus niger* with a large number of carbon compounds has been made at the same carbon level (112). The yields in grams per gram of carbon supplied were: D-glucose, 1.43; D-mannose, 1.46; D-fructose, 1.38; L-sorbose, 1.26; and D-xylose, 1.09. Other compounds gave much poorer growth. Enolization was not a factor in sugar utilization. All pentoses and hexoses having an L-3 carbon atom and a D-4-carbon atom were assimilable except the epimers of D-xylose and probably L-sorbose. These configurations are identical with those of the alpha and beta carbon atoms of the natural β-hydroxy amino acids.

Apparent exceptions are numerous, however, to the above generalizations. D-mannose could not be used by *Achlya* sp., *Isoachlya anisospora, Saprolegnia delica;* nor sucrose by *I. anisospora* and *S. monoica* (8). *Coprinus lagopus* is reported to require cellulose and to be unable to use hexoses (128). The majority of fungi respond approximately as does *A. niger: Phymatotrichum omnivorum* (9), *Rhizopus suinus* (21), *Stereum gausapatum* (46), *Venturia inaequalis* (57), and *Allomyces Kniepsii, Blastocladiella variabilis, Rhizophilyctus* sp. (86). *Leptomitus lacteus* (105) cannot use sugars.

The problem of preventing molding of fabrics has led to increased study of cellulose decomposition capacities of the fungi. Extensive surveys of large numbers of fungi have been published (54, 71, 131). The effect of nitrogen nutrition on cellulose decomposition of *Trichoderma viride* (10) and of *Coprinus lagopus* (128) has also been studied to some extent.

The utilization of amino acid carbon at an identical level has been studied with *Aspergillus niger* (111). The highest utilization factors obtained were L-proline, 0.22; L-aspartic acid, 0.21; L-glutamic acid, 0.23; L-ornithine, 0.10; and L-arginine, 0.10. A mixture of L-proline, L-glutamic acid and L-ornithine had a carbon utilization factor of 0.82 as compared to that of 1.17 for sucrose. The utilization of carbon in this mixture was not improved by addition of DL-alanine, L-aspartic acid, glycine or L-hydroxyproline. It appears probable therefore that this fungus is capable of using a mixture of its amino acids previously formed from sugar almost as efficiently as hexose in the elaboration of the many compounds needed for its growth and development. Some evidence was obtained, however, that substitution of the hydroxy

for the amino acids was still more effective (112) in supporting growth at highest efficiency. Roine (97*a*) reached a similar conclusion on the basis of chemical studies with yeast, concerning primacy of dicarboxylic amino acids, particularly glutamic, in amino acid syntheses from ammonium nitrogen. Results and conclusions almost identical with those published for *A. niger* were recently reported for yeasts (106*a*) also.

Similar investigations have been made with *Fusarium oxysporum* var. *lycopersici* and *Penicillium roquefortii* (39). Of the 25 compounds tested, 17 permitted growth with amino acid carbon in 0.5% concentration. Growth was estimated visually. *Aspergillus oryzae* (125) was grown on amino acids as sole source of both carbon and nitrogen. Yields were reported to rank as follows: isoleucine > alanine > glutamic acid > arginine, phenylalanine > histidine > tryptophane, valine, proline > glycine, serine, aspartic acid > lysine. Respiration ranked in this order: alanine > isoleucine, phenylalanine, tryptophane > leucine, serine, valine > proline, histidine, glycine > arginine, glutamic acid > aspartic acid > lysine. It is evident that growth and respiration are not identically parallel phenomena as concerns amino acids at least.

The re-elaboration of respiratory carbon dioxide by *Aspergillus niger* was again demonstrated and its inhibition found to enhance the effects of micronutrient deficiencies (110). The conclusion was reached with *Rhizopus nigricans* in shake cultures that carbon dioxide utilization by fungi was less than that attained by propionic acid bacteria (25). Radioactive carbon of carbon dioxide was largely localized in the acid-insoluble material of the fungus and in the carboxyl groups of formic and citric acids.

Respiratory studies with carbon compounds have also been made with *Penicillium notatum* (132) and *Oospora lactis* (136). Rate of oxidation in the latter organism (glucose > xylose > arabinose) appeared to parallel yields in *Oospora*. The data for *Penicillium* showed several divergencies, however, in that galactose was as readily oxidized as glucose or mannose, while sucrose, fructose and xylose were no better than arabinose. The accumulation of yield and respiration data for a large number of compounds and fungi would appear to be most desirable for an understanding of carbon metabolism.

Failure to grow on diose, triose, etc. sugars has usually been

interpreted as signifying that the fungus was lacking in sucrase, maltase, lactase, diastase and so on. Estienne, Castagne and Bertrand (24) examined a large number of fungi for ability to grow on lactose and tested the powdered fungi for lactase. They reported that high yields on lactose were not paralleled by high lactase content of the organism. They found strain differences to exist and little or no lactase in the higher fungi.

A brief article by Caputto *et al* (18) on galactose utilization by *Saccharomyces fragilis* is of special interest to the writer. Yeasts can be "trained" to ferment this sugar. Lindegren (65) has found that they may also be bred for ability to employ galactose. It appears now that *S. fragilis* produces galactokinase which catalyzes transphosphorylation between adenosine triphosphate and galactose. The reaction product, galactose-1-phosphate, was transformed to glucose-6-phosphate. Apparently the hexose-amino acid conversion reaction in yeasts using galactose is probably the same as in non-galactose using strains.

NITROGEN REQUIREMENTS

The generalization that fungi capable of using nitrate-nitrogen can also use ammonium-nitrogen (108) appears not to be without apparent exceptions. *Alternaria solani* (78) does not grow well on ammonium-nitrogen as compared to nitrate-nitrogen. On the other hand, of 14 species of *Marasmius* all could use ammonium-nitrogen and only one nitrate-nitrogen also (61). A few other fungi reported to thrive on ammonium-nitrogen include *Achlya* sp., *Isoachlya anisospora* var. *indica*, *Saprolegnia delica*, *S. monoica* and *Brevilegnia gracilis* (7). Only the last could also use nitrate. *Ophiobolus graminis* can utilize nitrate (35). Ammonium is used by *Collybia dryophila* (62), *Cenococcum graniforme* (73) and yeast (79, 107).

Good growth has been reported in synthetic media supplied with inorganic nitrogen by *Trichoderma viride* (10), *Sclerotinia sclerotiorum* and *Alternaria solani* (78), *Saccharomyces cerevisiae* (80), *Phacidium infestans* (81), *Memnoniella echinata* and *Stachybotrys atra* (82), *Sclerotinium delphinii* (83), *Pythium arrhenomanes*, *P. deliense*, *P. graminicolum* and *P. mamillatum* (99), *Saprolegnia delica* (102), *Aspergillus oryzae* (125) and *Phycomyces blakesleeanus* (12).

Asparagin was a good source of nitrogen for *Leptomitus lacteus* and *Apodachlya brachynema* in the presence of acetate, but neither could use inorganic nitrogen (104), and for *Penicillium digitatum* (134). Forms growing poorly or not at all on inorganic nitrogen included *Blastocladia Pringsheimii* (17), *Lactarius deliciosus* (74) and *Pythium indigoferae* (101). A mixture of amino acids could not replace peptone for the last organism. A large number of fungi causing decay of living oaks were successfully cultured on malt agar (22), but no attempt to use synthetic media was made.

Growth responses of fungi to amino acids fall into two categories, specific and non-specific. *Blastocladia Pringsheimii* (17) requires methionine but is aided in growth by supplementation of its solution with still other amino acids. Amino acids increased the growth of *Cenococcum graniforme* when grown on ammonium-nitrogen (73), and of *Lactarius deliciosus* (74).

The amino acid compositions of *Saccharomyces cerevisiae, Rhodotorula rubra, Rhizopus nigricans, Aspergillus niger* and *Penicillium notatum* have been determined by Stokes and Gunness (124). Histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, phenylalanine and tryptophane were estimated. The authors concluded that the amino acid compositions are qualitatively and quantitatively a stable and characteristic property of the cell under fixed conditions of growth. The fungal mycelia contained 50% more protein prior to sporulation than after sporulation. The individual amino acid content was found to vary with environmental factors, such as the medium, aeration; and with age.

Quantitative studies of amino acid utilization must be interpreted on the basis of type of nitrogen assimilation employed by the fungus. One that is unable to use inorganic nitrogen may be assumed to employ the amino acids as such. Another that can use inorganic nitrogen may make use only of ammonium-nitrogen subsequently to deaminizations. *Aspergillus niger* and the yeasts fall into the latter group.

A quantitative study of the growth of *A. niger* with amino acids (111) disclosed that these fell into three groups. Nitrogen in alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, ornithine and proline proved fully equivalent to inorganic nitrogen. Serine, threonine and tryptophane gave yields approxi-

mating 50% nitrogen assimilation. The balance of the amino acids gave very poor growth. It was pointed out that none of the first group contained a stable cyclic group or branched carbon chain, nor were they among those incapable of synthesis by the rat. It was further pointed out that none of the amino acids except three of the eight in the first group could also be used as a source of carbon supply by the fungus. Glutamic acid, ornithine and proline are considered for these and other reasons to be precursors of other amino acids.

Nielsen (79) in similar experiments with yeast found practically complete utilization of amino nitrogen in the same eight amino acids and in leucine and tyrosine also. The reason for failure to obtain complete agreement with the results when using *A. niger* may be associated with the use of beer-wort instead of the pure vitamins. Schultz and Pomper (107) studied utilization of amino-nitrogen by 19 species in six genera of yeasts. Excellent growth was obtained with five of the above eight amino acids (hydroxy-proline, ornithine were omitted) in 16 or 17 of the yeast species. Glycine gave good growth with only five species, and tyrosine with seven species. Leucine nitrogen was effective with 16 species.

Growth and respiration of *Leptomitus lacteus* (104) and respiration of *Penicillium chrysogenum* (133) with amino acids have also been investigated. The results showed both similarities and differences to the growth data obtained with *Aspergillus niger*.

An interesting observation by Robbins and McVeigh (97) is that *Trichophyton mentagrophytes* is quite sensitive to 50 p.p.m. of L-hydroxyproline. This amino acid gave a similar response with *T. gypseum, T. purpureum, Epidermophyton flocculosum* and *Microsporum canis*. Nineteen other species were not inhibited in growth under these conditions. In the writer's view these results afford an interesting demonstration of contrasting action of amino acids in the free as compared to the bound condition in proteins. This response to L-hydroxyproline is also given by tobacco seedlings which are killed at a concentration of five p.p.m. (116).

MINERAL REQUIREMENTS

Studies of the mineral nutrition of fungi have progressed relatively slowly since the previous review in this journal. Perlman (84) has recently reviewed the new developments with respect to

micronutrients but has concerned himself to a great extent with the metabolic products of fermentation. Since such studies have as their object the maximum production of a metabolite, adjustment of the components of the nutrient solution are in general opposed to optimum growth (26). It is not improbable that the unbalanced nutrient solutions employed in fermentation may at times lead to a distorted and erroneous concept of normal metabolism.

The writer has published a brief summary of investigations on mineral nutrition with *Aspergillus niger* (113). Emphasis was placed on the fact that the quantity of each chemical element required for maximum growth was exactly proportional to the quantity of sugar in the medium. The minimum quantity of macronutrient salts per gram of yield was determined to be 100 mg. or 10.6% of the sugar present. The solution contained ammonium nitrate, potassium phosphate and magnesium sulfate and was adjusted to exact quantity with potassium nitrate and ammonium sulfate. Micronutrients were added in tested optimum quantities: iron, zinc, copper, manganese, molybdenum and gallium. Each element was present in minimal amount corresponding to individual determinations of its optimum for growth (114).

The specificity of potassium and magnesium for nutrition of *A. niger* (115) was checked by substituting lithium, sodium, rubidium and cesium for potassium, and beryllium, calcium, strontium and barium for magnesium. Only sodium and beryllium were even slightly effective and then only when potassium or magnesium, respectively, were 50% or more deficient. *A. niger* could assimilate sulfate, sulfite and sulfoxylic acid equally efficiently but could not use sulfide or disulfide. Alkyl sulfonates and sulfinates were equally effective as were cystine and methionine sulfur (109).

Laborey (55) determined the magnesium/phosphorus absorption ratio and concluded it to be a constant. The Mitscherlich equation was found to give good agreement with yields for magnesium, phosphorus and potassium. Magnesium absorption agreed with the formula $KX^{1/P}$ where K was a constant, $^{1}/_{P}$ a constant less than one, and X the concentration of magnesium per 100 ml.

Most investigators have limited themselves in their work to the macronutrient elements enumerated under *A. niger*. The relatively few studies dealing with the known micronutrients included

work with a yeast (53) found to require iron and zinc; *Phycomyces blakesleeanus* (58, 59); some of 14 species studied in the genus *Marasmius* were concluded to require zinc, manganese and calcium (61); *A. niger* could not be demonstrated to need manganese under other than acid conditions (68); the necessity of molybdenum for *A. niger* was again verified (76). *Phythiomorpha gonapodyides* and *A. niger* required more zinc and manganese than *Phycomyces blakesleeanus* (88).

A few investigations dealt with elements not hitherto demonstrated to be essential for any of the fungi. In a series of carefully executed experiments *A. niger* was found to increase 20.9% in yield through addition of vanadium (5). Vanadium was present in all fungi examined (6) in 0.15 to 156 p.p.m. A comparison of the need for calcium by various fungi was made in quartzware with spectroscopically pure chemicals and led to the conclusion that certain fungi required two to four p. p.m. of calcium in their nutrition (118). *Aspergillus niger* and *Fusarium oxysporum* were not diminished in yield by omission of calcium. *Rhizoctonia solani, Sclerotium rolfsii, Cercospora nicotianae, Pythium irregulare* and *Thielaviopsis basicola* decreased in yield when calcium was not added. *Rhizoctonia* decreased 85.7% in yield on omission of calcium, nor could any of a series of trace elements replace its action. Boron, zinc and manganese were found necessary for growth of *Fusarium vasinfectum, F. udum* and *F. moniliforme* when using the dithizone technique (135).

The not too distant future should provide information concerning the need of additional biologically essential elements by fungi. Too little use has been made of the calcium carbonate method for removal of trace element impurities and of the dithizone reaction for confirmation of their absence in the reagents. A paper on the preparation of chemicals free of trace element impurities for use as spectroscopic standards (52) verifies the biological data obtained with the carbonate method. A tube method of measuring growth in trace element and vitamin studies has been proposed (98). A new simple technique for preparation of silica gel media (126) from a commercial colloidal silica preparation should facilitate the study of fungi requiring solid media. No reports are as yet available concerning the application of 8-hydroxyquinoline for removal of molybdenum (47) in fungal nutrition studies. Additional light

is thrown on the difficulty of freeing nutrient media containing amino acids from trace elements by a recent article (77).

The presence of cobalt in vitamin B_{12} and the probability of its synthesis only by bacteria would indicate the desirability of reaching higher levels of purity in micronutrient studies with fungi. A wide variety of forms having different requirements may be found to have specialized needs for trace elements. Those which the writer has studied, however, include only *A. niger* and fungal pathogens of the tobacco plant, and were found to have much the same needs for iron, zinc, copper, manganese and molybdenum.

VITAMIN REQUIREMENTS

A large number of papers have appeared in the past decade on the vitamin and other accessory compounds required by fungi. Details of these studies can be obtained in a book by Schopfer (106) and an excellent review by Robbins and Kavanagh (90). The earlier work has been amply verified and extended to demonstrate that vitamin and similar deficiencies are of very frequent occurrence among fungi. The fact that many of those successfully cultured grow very slowly would indicate, however, that many factors of this nature are still unidentified.

The compounds most frequently deficient in culture are thiamin, biotin and pyridoxine, though inositol and hypoxanthine are also needed in some cases. Yeasts (14) and presumably other fungi may require pantothenic acid and nicotinic acid. Vitamins A, D, E and K, riboflavin, ascorbic acid, folic acid and B_{12} which contains cobalt have not been reported as indispensable for any of the fungi. The data of metabolic deficiencies in mutant strains produced by chemical, ultra-violet and X-ray induction would indicate the possible occurrence of many other organic deficiencies. These include among other substances amino acids, purines, pyrimidines, nucleotides and choline.

No attempt will be made to list the vitamin requirements of all fungi reported upon. Many of the forms mentioned in the sections on carbon and nitrogen requirements cannot grow in synthetic media without vitamins. Only a few of the more recent publications are here included.

Sclerotinia camelliae (3) was found to require biotin, thiamin and inositol; *Graphium ulmi* (13), pyridoxine; *Schizosaccharo-*

myces pombe (20), biotin, nicotinic acid, calcium pantothenate, inositol and thiamin; *Melanospora damnosa* (38), biotin and thiamin; *Collybia dryophila* (62), thiamin and biotin; *Memnoniella* sp. and *Stachybotrys* sp., biotin (70); *Fusarium avenaceum*, biotin (91); *Phycomyces blakesleeanus*, thiamin and hypoxanthine (89); *Ceratostomella ulmi*, pyridoxine (93); *Trichophyton discoides*, pyridoxine, inositol, thiamin and as yet unknown compounds (96); *Ophiostoma cato nianum*, *Ceratostomella ips*, *C. microspora*, *C. montium*, *C. annulata* and *C. ulmi* all required pyridoxine (94, 95); *Pythium arrhenomanes*, *P. deliense*, *P. graminicolum*, *P. hyphalostricton* and *P. mamillatum* required thiamin (100); and *Ophiobolus graminis*, thiamin and biotin (130).

Nine species of *Ophiostomella* (*Ceratostomella*) were investigated by Fries (29). *O. piceae*, *O. stenoceras*, *O. coeruleum*, *O. quercus*, *O. pini* and *O. multiannulatum* needed thiamin. *O. pini* also required biotin, and *O. multiannulatum*, pyridoxine. Only pyridoxine was indispensable for *O. ulmi*, *O. fagi* and *O. filiferum*. *Mitrula paludosa* needed biotin and thiamin with nitrogen supplied as ammonium salt, but good growth was attained without biotin on nitrates. A series of eight *Coprinus* species required thiamin, usually only the pyrimidine moiety sufficing (27).

Litter-decomposing Hymenomycetes (34 species, 13 genera) were found usually to require thiamin (63), while need of other substances was rare on glucose and ammonium-nitrogen agar. Mycorrhizal (tree) fungi frequently required thiamin and biotin, but no uniformity was found to exist (75). A group of ten species of *Ceratostomella* were found to suffer from partial to complete deficiencies of biotin, thiamin and pyridoxine (92).

Torulopsis utilis synthesized more thiamin, riboflavin, nicotinic acid and pyridoxine in cultures deficient in iron; and less biotin, inositol and p-aminobenzoic acid (60). Pantothenic acid synthesis was not affected, according to Lewis. Meissel (72) found thiamin injurious to *Endomyces magnusii* in the absence of biotin.

REPRODUCTION

It is almost traditional in botany to consider growth and reproduction as two opposing phenomena and that one must predominate at the expense of the other. Little mention is made in

this connection, however, of everblooming plants in which both processes continue side by side. In the writer's opinion the major bases for this belief are those cases in which supplies of metabolites are insufficient for both processes. A contributing phenomenon is the fruiting of fungi when transferred to water or dilute solutions—but only probably when excessive quantities of nutrients have been employed in the medium. As concerns homothallic fungi grown in synthetic solutions, reproduction should be viewed as a normal and inevitable process consequent on normal preliminary growth.

The work of Coons, in which emphasis was placed on the use of dilute solutions as culture media, has been given a theoretical basis by certain observations made by Hawker (43). Optimum sugar concentrations for perithecia formation in *Melanospora destruens* (42) were lower than for growth, and increased growth substances increased the sugar optimum for perithecia formation. Both scarcity and excess of nutrient supplies may therefore prevent reproduction and presumably within limits fall within those for growth.

Improved sporulation because of added vitamins has frequently been observed. Thiamin was effective with *Ceratostomella fimbriata* (2) and biotin with *Sordaria fimicola* (1) and *Chaetomium globosum* (15) in perithecia formation. Biotin increased conidial production in *Memnoniella echinata* (15). Both biotin and thiamin were needed for formation of perithecia in *Melanospora damnosa* (38). Hexose phosphates increased perithecia in *Melanospora destruens* (44); thiamin that of *Pellia epiphylla* (69); and biotin, thiamin and pyridoxine for some of eight species of *Ceratostomella* investigated (92). Other studies on the nutritional factors of reproduction include those by Butler, Robbins and Dodge (16), Hawker (41), Hawker and Chaudhuri (45), Leonian and Lilly (59), Richards (87), Robbins and Ma (94), Voderberg (128) and Westergaard (129).

Too little attention has been paid to the possible influence of high solar light intensities on reproduction. Many fungi causing leaf diseases grow and fruit normally in bright sunlight. *Diaporthe phaseoli* from soybean (127) formed an increased number of perithecia under fluorescent lamps, even though no asci or ascospores were produced.

MUTATIONS

A great deal of evidence had accumulated in the older literature concerning instability and degeneration of cultures (19). A series of papers by Steinberg and Thom (120–122) verified previous claims for chemical induction of mutation in *Aspergillus* and suggested that some mutations might be due to alterations in cell proteins (chromatin, enzymes, etc.) of the fungus. "Injury mutants" showing loss of physiological capacities and morphological abnormalities were obtained from a single spore strain of *Aspergillus niger* and perpetuated unchanged. Other stable strains of *Aspergillus* gave identical results. Chemical induction of mutations was brought about by addition of sodium nitrite to a complete and neutral medium containing ammonium-nitrogen, so that acidity steadily increased during growth of the fungus. Heterokaryosis (40) was not a factor in these results, since a single spore presumably containing only a single haploid nucleus had been used to start the *A. niger* strain. These strains had maintained themselves unchanged for more than a quarter of a century under routine conditions. The mutants of *A. niger*, however, could not be tested through the sexual stage.

More recently, however, the genetic investigations by Beadle and his associates (4) would appear to substantiate the prevailing opinion that actual mutations were taking place. *Neurospora* spores mutate under X-rays, and if the least modified of these are selected and crossed, the states of physiological injury (thiaminless, arginineless, etc.) are transmitted through the sexual stage on a one-gene-one injury basis. Alterations in the enzymes of the cell also appear to play a part in the changed nutritive capacities of *Neurospora* mutants.

The results of investigations by Fries (30, 31) with mutants of *Ophiostoma multiannulatum* have a similar import. Hyphal cells of this fungus are uninucleate, unlike *Neurospora*, and no evidence of heterokaryosis could be found (34). Normal cultures produced 0.03% mutants, and starvation cultures, 0.25 to 0.41% (32). Results of other estimates (33) were X-rays, 1.8% mutation; nitrogen-mustard, 1.3%; normal $< 0.06\%$; caffeine, 1%; and theophylline, 0.6%. *Neurospora crassa* (49) also mutated with nitrogen mustard-control, 0.13%; nitrogen mustard, 3.8%.

Numerous instances of reversion of mutants in cultures have

been reported. More recently these include *Aspergillus niger* (121, 122), *Ophiostoma multiannulatum* (31), *Neurospora crassa* (37), yeast (65, 66) and *Neurospora sitophila* (123). The chemical basis for reversion of cultures is still undetermined. Steinberg and Thom (122), as already mentioned, suggested that it consists of repair of chemical injury to cell protein, whereas others are inclined to believe the gene is produced anew. Other explanations may also exist.

A decision between repair and reconstruction of genes in reversions should take into consideration the fact that the gene may be present intact but modified to require altered conditions of operation. A "perithecia-less" *Aspergillus amstelodami* produced by nitrite fruited profusely with double the normal sucrose content (unpublished data of Steinberg and Thom). A pyridoxineless strain of *Neurospora sitophila* grew normally on an acid medium with ammonium-nitrogen (123). *Mitrula paludosa* required biotin with ammonium salt but grew slowly without biotin on nitrate (28). Sartory, Sartory and Wurtz (103) claim production of perithecia, asci and ascospores in *Aspergillus niger* under the influence of radium radiation.

The practical problem awaiting solution is the production and maintenance of stable strains of fungi for nutrition and pathogenicity studies. Studies by the writer on the mineral requirements of fungal pathogens of tobacco (118) had been suspended because of unavoidable degeneration of stock cultures on potato-dextrose agar. They became almost unrecognizable in appearance and almost incapable of growth or pathogenicity. Selection (mass) and propagation on an *Aspergillus niger* optimum solution fortified with six vitamins led to "rejuvenation" as concerned vigor and appearance. *Phytophthora parasitica* var. *nicotianae*, *Bacterium solanacearum*, *B. tabacum* and *B. angulatum* could also be maintained thereon. Its formula per liter was: sucrose, 50 gm.; NH_4NO_3, 1.90; KH_2PO_4, 0.55; $MgSO_4.7H_2O$, 0.35; agar, 12.5; biotin, 0.02 p.p.m.; thiamin, 1.0; niacin, 1.0; pyridoxine, 0.5; calcium pantothenate, 0.5; p-aminobenzoic acid, 0.10. Calcium carbonate was usually added to the tubes before autoclaving except those for *Sclerotium*. A small quantity of glutamic acid increased vigor of the bacterial cultures, *T. basicola* and *P. parasitica*. Trace elements were added to this medium but were probably unneces-

sary because of impurities in the agar. All cultures except those of *Phytophthora* remained viable after four months at 30° C. with parafilm seals.

PATHOGENICITY

A brief but excellent review of this subject has been published by Brown, Brooks and Bawden (11), and in a book by Gäumann (36). The citations that follow deal primarily with a few special products of exosmosis of fungi. No references are included to numerous studies of other products such as enzymes, peptides and acids of fermentation.

Sclerotium rolfsii (48) forms oxalic acid in sufficient quantity to kill the cells of its host. *Helminthosporium victoriae* produced a toxin in culture and on its host, and *Puccinia coronata* one on its host only (67). That of *H. victoriae* produced the same symptoms on its host as does the fungus. It was therefore concluded that susceptibility was induced by toxins. Toxin formation has also been reported for *Ceratostomella ulmi* (137) and *Phytophthora cactorum* (50, 51). Symptoms of Dutch elm disease—*Graphium ulmi*—could be produced by toxins formed in culture (23). Lycomarasmin from *Fusarium persici* caused wilt in the tomato plant. On hydrolysis it yielded glycine, dl-aspartic acid, pyruvic acid and ammonia but no serine (85).

Spores of *Piricularia oryzae* grown on a synthetic medium were found to be as virulent as those grown on the rice plant (56). Leben and Keitt (57) studied the nutrition of four pathogenic and three non-pathogenic strains of *Venturia inaequalis,* the apple-scab fungus. They reached the conclusion that no differences in carbon, nitrogen or vitamin nutrition of the various strains were clearly associated with their pathogenic capabilities.

Availability of stable strains of pathogenic fungi producing toxins should permit of studies relating pathogenicity to nutrition in some of its phases. It is well known that enzymes, amino acids, peptides and organic acids are among the products of exosmosis of fungi. The marked toxicities of amino acids to tobacco seedlings (116, 119), and even of diffusates from non-pathogenic soil bacteria (117), may prove to be an important factor in pathogenicity.

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